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Screening of the two-component-system histidine kinases of *Listeria monocytogenes* EGD-e. LiaS is needed for growth under heat, acid, alkali, osmotic, ethanol and oxidative stresses

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ABSTRACT

To study the role of each two-component system (TCS) histidine kinase (HK) in stress tolerance of *Listeria monocytogenes* EGD-e, we monitored the growth of individual HK deletion mutant strains under heat (42.5 °C), acid (pH 5.6), alkali (pH 9.4), osmotic (6% NaCl), ethanol (3.5 vol%), and oxidative (5 mM H_2O_2) stresses. The growth of Δ*liaS* (Δ*lmo1021*) strain was impaired under each stress, with the most notable decrease under heat and osmotic stresses. The Δ*virS* (Δ*lmo1741*) strain showed nearly completely restricted growth at high temperature and impaired growth in ethanol. The growth of Δ*agrC* (Δ*lmo0050*) strain was impaired under osmotic stress and slightly under oxidative stress. We successfully complemented the HK mutations using a novel allelic exchange based approach. This approach avoided the copy-number problems associated with *in trans* complementation from a plasmid. The mutant phenotypes were restored to the wild-type level in the complemented strains. This study reveals novel knowledge on the HKs needed for growth of *L. monocytogenes* EGD-e under abovementioned stress conditions, with LiaS playing multiple roles in stress tolerance of *L. monocytogenes* EGD-e.

1. Introduction

Listeria monocytogenes is a Gram-positive, non-spore-forming bacterium widely distributed in nature and capable of persisting in food-processing plants for years (Gray and Killinger, 1966; Keto-Timonen et al., 2007; Miettinen et al., 1999; McLauchlin and Rees, 2009; Tompkin, 2002). It is the causative agent of relatively rare but life-threatening listeriosis, with mortality of up to 20–30% among risk groups (Centers for Disease Control and Prevention, 2014; Schlech et al., 1983; de Valk et al., 2005). One of the key factors that make *L. monocytogenes* a notable threat for food safety is its ability to overcome extreme stress conditions. The maximum growth temperature of *L. monocytogenes* reaches 45 °C (Gray and Killinger, 1966). *L. monocytogenes* grows at a pH as high as 9.6 (Gray and Killinger, 1966), while the minimum pH required for initiation of its growth varies from 4.3 to 5.2 at 30 °C (Farber et al., 1989). In addition, *L. monocytogenes* grows even in 10% NaCl (Gray and Killinger, 1966) and in water activity down to 0.90 (Nolan et al., 1992). As for ethanol, minimal inhibitory concentration is 5% (Oh and Marshall, 1993; Cotter et al., 1999). *L. monocytogenes* also grows without problems in 0.025% hydrogen peroxide (Kallipolitis and Ingmer, 2001).

Two-component regulatory signaling systems (TCSs) aid bacteria in adapting to arduous environmental stress factors (Stock et al., 2000; West and Stock, 2001). In the sequenced genome of the wild-type *L. monocytogenes* strain EGD-e, genes encoding 16 TCSs have been recognized, of which DegU (Lmo2515) is an orphan response regulator (RR) and others are complete TCSs (Glaser et al., 2001; Williams et al., 2005). Knowledge on RRs in stress tolerance of *L. monocytogenes* is available in abundance compared to histidine kinases (HKs), although some differing results have been displayed. Previously, Williams et al. (2005) assessed roles of each RR of *L. monocytogenes* EGD-e at high temperature (43 °C), in NaCl (9%)

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and under oxidative (0.025% H_2O_2) and ethanol (5%) stresses. Consequently, ethanol stress lead to decreased growth of RR deletion mutants Δ*virR* (Δ*lmo1745*), Δ*resD* (Δ*lmo1948*) and Δ*phoP* (Δ*lmo2501*) and to completely restricted growth of Δ*degU* (Δ*lmo2515*) (Williams et al., 2005), while other studied stress conditions did not alter the growth of the mutant strains in comparison to the wild-type EGD-e (Williams et al., 2005). However, by insertional mutagenesis, Kallipolitis and Ingmer (2001) found also KdpE (Lmo2678) and CesR (Lmo2422) to contribute to growth of *L. monocytogenes* LO28 under heat (43.5 °C) and NaCl (9%) stresses, and LisR (Lmo1377) to heat and oxidative (0.025% H_2O_2) stress tolerance and to acid tolerance (pH 5.25) at logarithmic growth phase. Brøndsted et al. (2003) further demonstrated by insertional mutation that KdpE contributes to osmotic stress tolerance of *L. monocytogenes* EGD (2% NaCl). Strikingly, deletion in *kdpE* did not lead to growth changes under the same stress condition (Brøndsted et al., 2003). Regarding osmotic stress, Garmyn et al. (2012) also found deletion in *agrA* (*lmo0051*) to result in significantly decreased growth of *L. monocytogenes* EGD-e in 3 and 5% NaCl, although no changes were detected at higher salt concentration (9% NaCl) by Williams et al. (2005). Lastly, in addition to the role of DegU under ethanol stress (Williams et al., 2005), Gueriri et al. (2008) found deletion in *degU* to lead to inability of *L. monocytogenes* EGD-e to grow at 44 °C.

Unlike RRs, HKs and their role in stress tolerance of *L. monocytogenes* have been far less studied. Thus far, contribution of each HK to cold tolerance of *L. monocytogenes* has been assessed (Pöntinen et al., 2015). In addition, few reports on individual HKs have been presented. LisK (Lmo1378) has been ascertained to play a role in osmotolerance (8% NaCl) of *L. monocytogenes* LO28 (Sleator and Hill, 2005) and in growth of the same strain at pH 3.5 during exponential growth (Cotter et al., 1999). In addition, Kallipolitis et al. (2003) found *cesK* (*lmo2421*) to contribute to ethanol sensitivity of *L. monocytogenes* LO28.

High temperature and acid, alkali, osmotic, ethanol, and oxidative stresses are widely used in preserving food products or in sanitizing food processing environments, and the ability of *L. monocytogenes* to surmount these stresses poses a notable risk to the food safety. Thus, it is necessary to unveil the various stress tolerance mechanisms utilized by *L. monocytogenes*. Due to limited knowledge available on the role of TCS HKs in the stress tolerance of *L. monocytogenes*, we identified by mutational analysis the HKs needed for growth of *L. monocytogenes* EGD-e under above-mentioned stress conditions.

2. Materials and methods

2.1. Strains and plasmids

L. monocytogenes wild-type strain EGD-e (Glaser et al., 2001), derived HK deletion mutant strains with each *L. monocytogenes* HK gene deleted at a time (Pöntinen et al., 2015), and the complementation strains *ΔagrC*c (Δ*lmo0050*c), Δ*liaS*c (Δ*lmo1021*c) and Δ*virS*c (Δ*lmo1741*c), and the plasmids and *Escherichia coli* strains used in this study are presented in Table 1. The *L. monocytogenes* strains were grown at 37 °C on blood agar, Brain-heart infusion (BHI) agar or broth (Oxoid, Cheshire, England) and the *E. coli* strains at 30 °C on Luria-Bertani (LB) agar or broth (Oxoid). Erythromycin (5 μg/ml) or ampicillin (100 μg/ml) (Sigma-Aldrich, St. Louis, MO) was added to BHI or LB, respectively, when needed.

2.2. Complementation

Complementation of notably stress-sensitive mutant strains, resulting in strains without an antibiotic resistance gene or an integrated conjugation plasmid, was performed by allelic replacement using a pMAD plasmid shuttle vector (Arnaud et al., 2004) according to the method of Markkula et al. (2012a) and Pöntinen et al. (2015) with following modifications. An insert, including the deleted coding sequence of the target gene, was amplified using outermost mutant strain construction primers −1 (MluI) and -4 (Table 2) and ligated into the MluI restriction site of pMAD. The vector with insert was then propagated in *E. coli* NEB5α electrocompetent cells (New England Biolabs, Ipswich, MA) and transformed into the corresponding *L. monocytogenes* deletion mutant strain by electroporation (25 μF, 200 Ω, 1.8 kV). Single-crossover colonies were selected at 39 °C in BHI broth with erythromycin and double-crossover colonies at 39 °C in BHI broth without antibiotic. Successful complementations were confirmed by PCR using gene-specific and outermost mutant construction primers (Fig. 1A and B).

2.3. Growth curve analyses

Growth curve analyses of the wild-type strain EGD-e, the mutants and complementation strains were carried out according to the method of Markkula et al. (2012a, b) using the Bioscreen C Microbiology Reader (Growth Curves, Helsinki, Finland). The strains were exposed to each stress factor separately as follows. Overnight inoculations of five individual colonies per strain were diluted (1:100) in BHI adjusted to pH 5.6 (HCl 37%) or 9.4 (5 M NaOH) or supplemented with 6% NaCl or 5 mM H_2O_2 (30%). Inoculations of four individual colonies per strain were diluted in BHI supplemented with 3.5 vol% ethanol (99.5%), in order to leave outermost wells blank for buffering evaporation of ethanol. Growth under heat stress was measured at 42.5 °C, while other growth curve analyses were performed at 37 °C. All the strains were grown for 48 h in 6% NaCl and 3.5% ethanol and 24 h under other stresses and the OD_{600} values were measured at 15-min intervals under each stress. The mean maximum growth rate of each strain was obtained using DMFit web edition software (Computational Microbiology Research Group, Institute of Food Research, Colney, Norwich, UK [http://browser.combase.cc/DMFit.aspx accessed latest at 26.11.2016]) using the model of Baranyi and Roberts (1994). The area under curve (AUC) values were calculated using a designed script in Matlab R2014a (MathWorks, Natick, MA), including interpolation function spline and integration function quad (adaptive Simpson quadrature) (Fig. S1). The statistical significance of the differences between AUC values and maximum growth rates of the mutant strains and those of the wild-type EGD-e strain was tested using independent samples 2-tailed *t*-test (IBM SPSS Statistics 23, IBM, Armonk, NY). The correspondence between the OD_{600} values and the viable cell numbers of the wild-type EGD-e strain, the deletion mutants and complementation strains at each stress were confirmed by plate counts at early and late logarithmic and early stationary growth phase.

2.4. Differences in maximum growth temperatures

The maximum growth temperatures of the wild-type EGD-e, all deletion mutants and the complementation strains Δ*liaS*c and Δ*virS*c were tested using Gradiplate W10 incubator (BCDE Group, Helsinki, Finland) according to the method of Markkula et al. (2012b). All the strains were grown for 48 h using a temperature gradient of 39–44 °C. The significances of the differences between the maximum growth temperatures were determined by 2-tailed *t*-test (SPSS).

3. Results

3.1. Phenotypes of L. monocytogenes HK deletion mutants

The growth of the HK deletion mutant strain Δ*liaS* (Δ*lmo1021*) was impaired under each stress condition studied compared to the wild-type EGD-e strain, with most notable changes at 42.5 °C and in 6% NaCl, moderate changes under both pH stresses (5.6 and 9.4) and with slight changes in 3.5% ethanol and 5 mM H_2O_2 (Fig. 2A–F). The maximum growth temperature of Δ*liaS* was 0.9 °C lower ($P < 0.001$) than that of the wild-type EGD-e strain (Fig. 3). Area under curve (AUC) values of Δ*liaS* were 31 and 30% decreased ($P < 0.01$) under osmotic and heat stresses, respectively, 21 and 15% decreased ($P < 0.001$) under acid and alkali stresses, respectively, and 11% decreased ($P < 0.01$) under both ethanol and oxidative stresses, compared to AUC values of the wild-type EGD-e under corresponding stress conditions (Table 3). The maximum growth rates of the Δ*liaS* strain presented 67% decrease ($P < 0.001$) in 6% NaCl and 15 to 46% decrease ($P < 0.01$) under other stress conditions compared to the wild-type EGD-e strain (Table 4).

The Δ*virS* (Δ*lmo1741*) strain showed almost completely restricted growth at 42.5 °C (Fig. 2A), and had 0.7 °C lower ($P < 0.001$) maximum growth temperature than the wild-type EGD-e strain (Fig. 3) and moderately impaired growth in ethanol (Fig. 2E). The AUC values of Δ*virS* strain were 58 and 19% decreased ($P < 0.01$) under heat and ethanol stresses, respectively (Table 3). The maximum growth rate of the Δ*virS* strain in ethanol was 33% decreased ($P < 0.01$), while its growth rate at high temperature was minuscule (Table 4).

The Δ*agrC* (Δ*lmo0050*) strain showed notably impaired growth at osmotic stress (Fig. 2D), with 33% decreased ($P < 0.01$) maximum growth rate compared to the wild-type EGD-e strain (Table 4). The Δ*agrC* strain also showed slight growth deficiency and 14% decreased ($P < 0.01$) AUC value under oxidative stress compared to the wild-type EGD-e strain (Fig. 2F, Table 3).

The Δ*agrC* and Δ*lmo2582* strains showed statistically significantly decreased growth parameters compared to the wild-type EGD-e strain under alkali stress (Fig. 2C, Table 4), as did the Δ*yycG* (Δ*lmo0288*) strain under ethanol stress (Fig. 2E, Table 3), Δ*resE* (Δ*lmo1947*) and Δ*lmo2582* strains under oxidative stress (Fig. 2F, Table 3), and several HK mutants at pH 5.6 (Fig. 2B, Tables 3 and 4). These observed decreases compared to wild-type strain values were, nevertheless, minor (less than 11%) (Fig. 2B, C and F, Tables 3 and 4), and these mutants were thus excluded from the complementation experiments. Other mutant strains showed no statistically significant decrease in AUC values or maximum growth rates compared to the wild-type EGD-e strain under any of the stress conditions studied (Tables 3 and 4) nor decreased maximum growth temperatures.

3.2. Complementation and phenotypes of complementation strains

The complementation successfully replaced the deletion area of the mutant strain with the wild-type copy of the corresponding target gene in the correct locus (Fig. 1A and B). The complementation of the HK mutants using the allelic exchange approach restored the wild-type phenotypes to the Δ*agrC*, Δ*liaS*, and Δ*virS* mutants that had shown significantly impaired growth under heat, acid, alkali, osmotic, ethanol or oxidative stresses (Fig. 4A–E). The complementation of the Δ*liaS* and Δ*virS* strains also restored their maximum growth temperatures to the wild-type level (Fig. 3).

4. Discussion

Importance of each HK in stress tolerance of *L. monocytogenes* EGD-e was studied by growth experiments of deletion mutant strains under heat, acid, alkali, osmotic, ethanol, and oxidative stresses. The Δ*liaS* strain showed impaired growth under each stress condition studied, with most notable changes under heat and osmotic and moderate changes under acid and alkali stresses (Fig. 2A–D, Tables 3 and 4), indicating multiple roles for the LiaS in stress tolerance of *L. monocytogenes*. The TCS LiaSR has been shown to respond to disturbances in cell envelope by remodeling cytoplasmic membrane composition (Fritsch et al., 2011). This could account for the role of LiaS in response to multiple stresses as environmental stress adaptation frequently requires sustenance of membrane functions and cell envelope structure (reviewed by Soni et al., 2011). Intriguingly, the cognate RR LiaR (Lmo1022) has not been allocated a role in stress tolerance of *L. monocytogenes* (Kallipolitis and Ingmer, 2001; Williams et al., 2005). However, LiaS has been proposed as a candidate for phosphorylating the orphan RR DegU (Williams et al., 2005). Indeed, LiaS is the only HK of *L. monocytogenes* harboring a group II kinase domain that is predicted to network with NarL-type RRs (Williams et al., 2005). Moreover, LiaR and DegU are the only RRs representing putative NarL-type in *L. monocytogenes* (Williams et al., 2005). This could explicate the fact that both LiaS (Fig. 2A, Tables 3 and 4) and DegU have a role in heat tolerance of *L. monocytogenes*, while LiaR does not seem to be involved (Gueriri et al., 2008; Williams et al., 2005).

The Δ*virS* strain showed almost completely restricted growth at high temperature (Fig. 2A, Tables 3 and 4) and a lower maximum growth temperature than the wild-type EGD-e strain (Fig. 3). However, the cognate RR VirR was not allocated with a role under heat stress (Williams et al., 2005). This could suggest that at high temperature VirS could phosphorylate one or several other RRs with already established role in heat tolerance. Indeed, of such RRs, LisR and KdpE harbor the same putative OmpR-type output domain as does VirR (Williams et al., 2005). In addition, VirS was suggested to cross-phosphorylate with one or more RRs by Mandin et al. (2005). Notably, cross-phosphorylation of the non-cognate TCS signaling pathways has been proposed (Laub and Goulian, 2007), and would be a noteworthy future research objective for divergent behavior of cognate HKs and RRs in stress tolerance of *L. monocytogenes*.

Impaired growth of the Δ*virS* strain in 3.5% ethanol (Fig. 2E, Tables 3 and 4) and the previously shown role for the cognate RR VirR (Lmo1745) under ethanol stress (Williams et al., 2005) suggest that the complete TCS VirRS plays a role in ethanol stress tolerance. Although titled by the same gene symbol, VirS of *L. monocytogenes* does not show any significant homology to the extensively studied VirRS system controlling toxin production in *Clostridium perfringens* (Lyristis et al., 1994). Instead, VirRS belongs to the BceRS-like TCSs, best described in *Bacillus subtilis* and suggested to be involved in the resistance against peptide antibiotics in *Firmicutes* bacteria (Dintner et al., 2011). In addition, the cognate RR VirR has been indicated to control virulence of *L. monocytogenes* by regulating genes involved in modification of surface components (Mandin et al., 2005). Changes in the cell envelope composition may, in turn, lead to enhanced ethanol tolerance, as was suggested by Williams et al. (2005).

The observed growth defects of Δ*agrC* in 6% NaCl (Fig. 2D, Table 4), together with a previous association of the cognate RR AgrA with response to 3 and 5% NaCl (Garmyn et al., 2012) strongly suggest an important role of the complete TCS AgrAC in osmotic stress. The genetic context of the locus encoding this TCS shows high homology to the locus encoding the extensively studied accessory gene regulator (Agr) quorum sensing system in staphylococci (Autret et al., 2003; Peng et al., 1988). In both *Staphylococcus aureus* and in *L. monocytogenes* EGD-e, the Agr system has been linked to virulence (Autret et al., 2003; reviewed by Novick and

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristic	Reference or source
L. monocytogenes		
EGD-e	Wild-type strain; serotype 1/2a	Glaser et al., 2001
Δ*lmo0050* (Δ*agrC*)	In-frame deletion of the *lmo0050* gene (1296 bp) with 1101 nucleotids	Pöntinen et al., 2015
Δ*lmo0288* (Δ*yycG*)	Out-of-frame deletion of the *yycG* gene (1833 bp) with 1682 nucleotids	Pöntinen et al., 2015
Δ*lmo0692* (Δ*cheA*)	In-frame deletion of the *cheA* gene (1857 bp) with 1752 nucleotids	Pöntinen et al., 2015
Δ*lmo1021* (Δ*liaS*)	In-frame deletion of the *lmo1021* gene (1059 bp) with 930 nucleotids	Pöntinen et al., 2015
Δ*lmo1061*	Out-of-frame deletion of the *lmo1061* gene (1446 bp) with 1249 nucleotids	Pöntinen et al., 2015
Δ*lmo1173*	Out-of-frame deletion of the *lmo1173* gene (1458 bp) with 1078 nucleotids	Pöntinen et al., 2015
Δ*lmo1378* (Δ*lisK*)	In-frame deletion of the *lisK* gene (1452 bp) with 1116 nucleotids	Pöntinen et al., 2015
Δ*lmo1508*	In-frame deletion of the *lmo1508* gene (1440 bp) with 1230 nucleotids	Pöntinen et al., 2015
Δ*lmo1741* (Δ*virS*)	In-frame deletion of the *lmo1741* gene (1041 bp) with 786 nucleotids	Pöntinen et al., 2015
Δ*lmo1947* (Δ*resE*)	In-frame deletion of the *resE* gene (1791 bp) with 1542 nucleotids	Pöntinen et al., 2015
Δ*lmo2011*	In-frame deletion of the *lmo2011* gene (1740 bp) with 1437 nucleotids	Pöntinen et al., 2015
Δ*lmo2421* (Δ*cesK*)	In-frame deletion of the *cesK* gene (1143 bp) with 1020 nucleotids	Pöntinen et al., 2015
Δ*lmo2500* (Δ*phoR*)	In-frame deletion of the *phoR* gene (1776 bp) with 1641 nucleotids	Pöntinen et al., 2015
Δ*lmo2582*	Out-of-frame deletion of the *lmo2582* gene (1380 bp) with 1339 nucleotids	Pöntinen et al., 2015
Δ*lmo2679* (Δ*kdpD*)	In-frame deletion of the *kdpD* gene (2691 bp) with 2652 nucleotids	Pöntinen et al., 2015
Δ*agrC*c	complemented strain	This study
Δ*liaS*c	complemented strain	This study
Δ*virS*c	complemented strain	This study
E. coli		
DH5α pMAD	DH5α strain containing the shuttle vector plasmid pMAD	Mattila et al., 2011
NEB5α	Electrocompetent strain	New England Biolabs
Plasmid		
pMAD	Cloning shuttle integration vector plasmid	Arnaud et al., 2004
pMAD- Δ*agrC*c	pMAD containing coding sequence of EGD-e *agrC*	This study
pMAD- Δ*liaS*c	pMAD containing coding sequence of EGD-e *liaS*	This study
pMAD- Δ*virS*c	pMAD containing coding sequence of EGD-e *virS*	This study

Table 2
Primers for complementation.

Gene	Primer name	Primer sequence (5′→ 3′)[a]	Reference
lmo0050 (*agrC*)	0050−1	NNNNNNGGATCCTTATGGCTCAGACGGTATTCT	Pöntinen et al., 2015
	0050−4	NNNNNNACGCGTCCACACCTTTTGTCGTATCT	Pöntinen et al., 2015
lmo1021 (*liaS*)	1021-1 MluI	NNNNNNACGCGTCGTAAGGAGGTGTGTCAGC	Pöntinen et al., 2015
	1021−4	NNNNNNACGCGTCACCAAATCGCCCAAGAC	Pöntinen et al., 2015
lmo1741 (*virS*)	1741-1 MluI	NNNNNNACGCGTGGTCAAAAACAACCCCGATA	Pöntinen et al., 2015
	1741−4	NNNNNNACGCGTAAGGCGGTAAGTTTGTTCCA	Pöntinen et al., 2015

[a] Restriction sites are underlined. N, any of the bases, i.e., adenine (A), cytosine (C), guanine (G), or thymine (T).

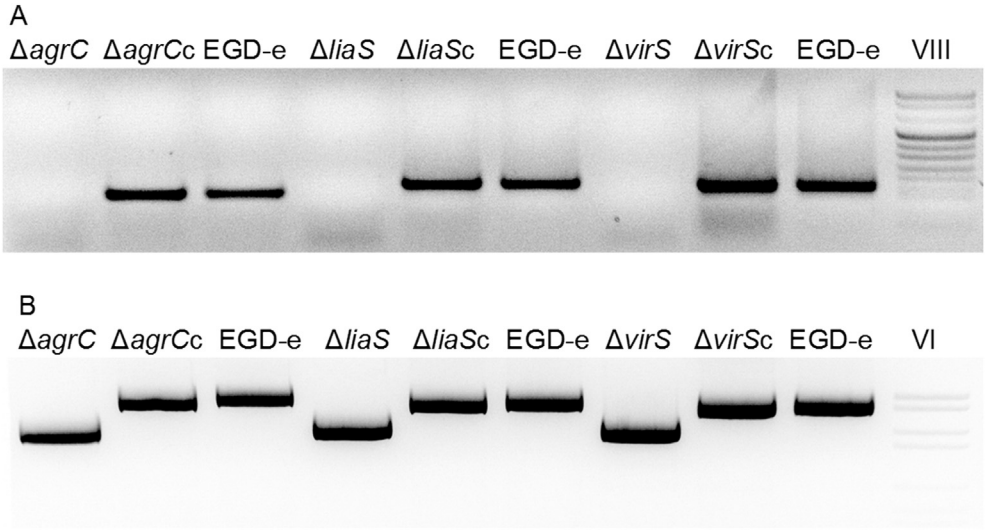

Fig. 1. Confirmation of complemented strains Δ*agrC*c, Δ*liaS*c, and Δ*virS*c using (A) gene-specific primers; (B) outermost mutant construction primers −1 (MluI) and -4. Wild-type EGD-e was used as a positive control and corresponding deletion mutant used as a negative control. Products of 30 cycles are shown. (VI and VIII) DNA Molecular weight markers VI (150−2100 bp) and VIII (19−1114 bp) (Roche Diagnostics, Indianapolis, IN).

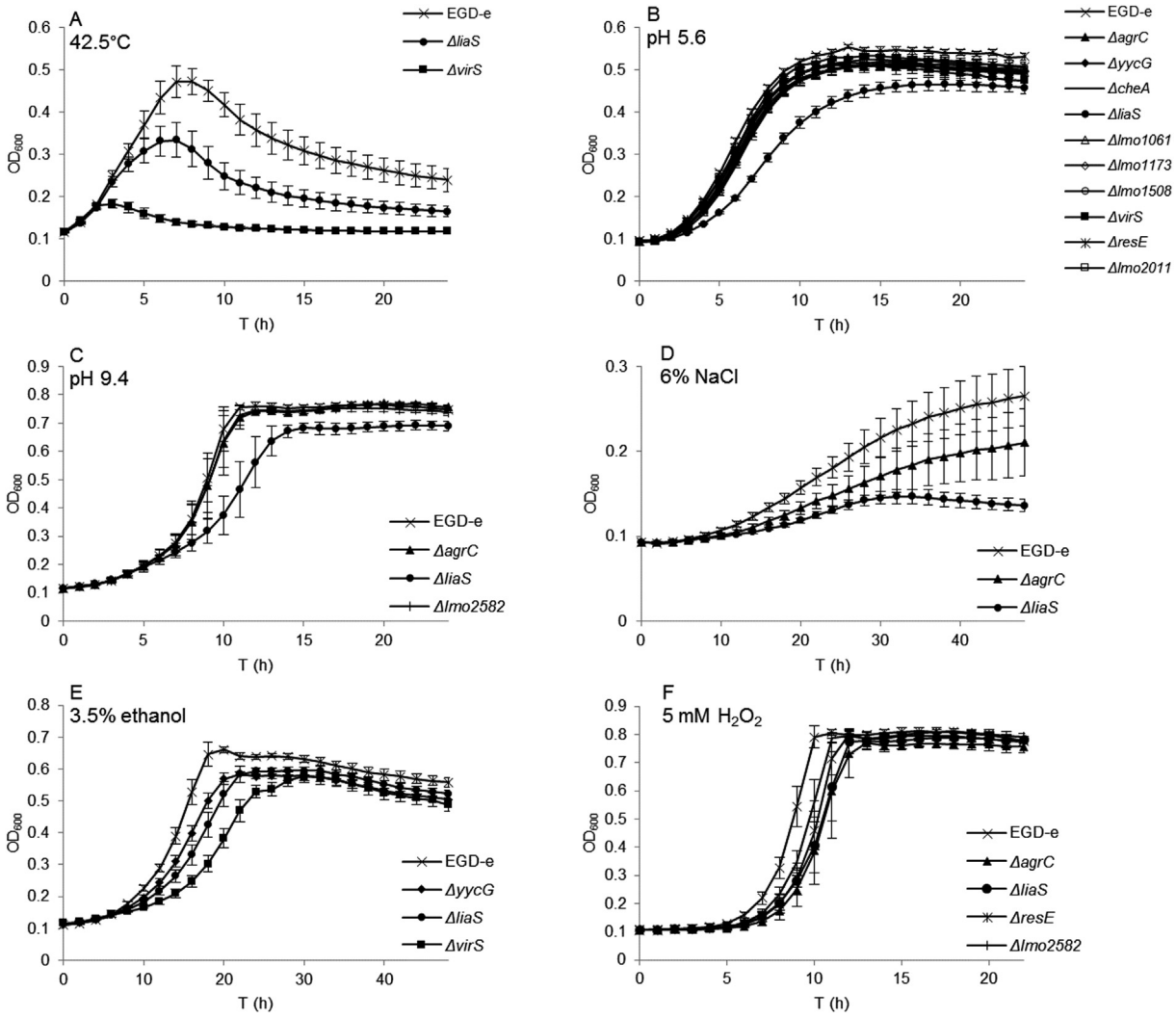

Fig. 2. Growth curves of *Listeria monocytogenes* wild-type EGD-e and the HK deletion mutant strains (A) Δ*liaS* and Δ*virS* in BHI broth at 42.5 °C; (B) Δ*agrC*, Δ*yycG*, Δ*cheA*, Δ*liaS*, Δ*lmo1061*, Δ*lmo1173*, Δ*lmo1508*, Δ*virS*, Δ*resE*, and Δ*lmo2011* at pH 5.6; (C) Δ*agrC*, Δ*liaS*, and Δ*lmo2582* at pH 9.4; (D) Δ*agrC* and Δ*liaS* in 6% NaCl; (E) Δ*yycG*, Δ*liaS*, and Δ*virS* in 3.5% ethanol; (F) Δ*agrC*, Δ*liaS*, Δ*resE*, and Δ*lmo2582* in 5 mM H_2O_2. All the strains were grown for 48 h in 6% NaCl and 3.5% ethanol and for 24 h in other stress conditions and the optical density at 600 nm (OD_{600}) was measured at 15-min intervals. The data shown represent the mean OD_{600} values and the error bars the standard deviation of four replicate cultures in 3.5% ethanol and five replicate cultures in other stress conditions.

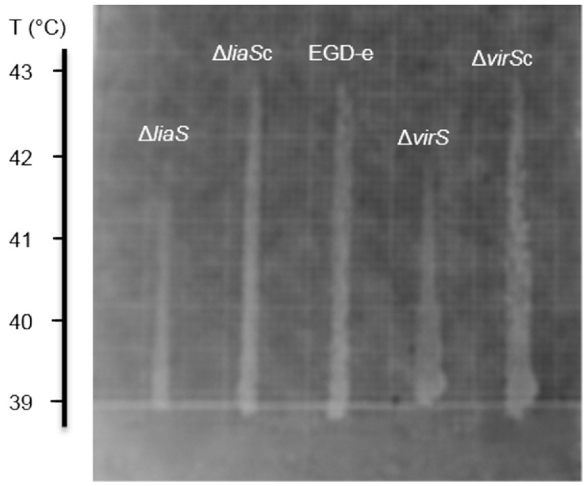

Fig. 3. Growth of *Listeria monocytogenes* wild-type EGD-e, the HK deletion mutant strains Δ*liaS* and Δ*virS* and the complemented strains Δ*liaS*c and Δ*virS*c in 2.5% tryptic soy agar within 48 h in the Gradiplate W10 incubator at a temperature gradient of 39–44 °C.

Geisinger, 2008). While the complete regulatory role of the Agr system in Listeria is yet to be elucidated, AgrAC mediated osmotic stress tolerance might be linked to the established role of AgrAC in virulence of *L. monocytogenes*. In fact, exposure to salt stress mimicking human intestinal conditions has been shown to increase virulence gene expression in *L. monocytogenes* (Sue et al., 2004).

Intriguingly, some HKs did not seem to be involved in the same stress tolerance, in which their cognate RRs have been demonstrated to have a role in. Besides the abovementioned cross-phosphorylation, some of these differences may be explained by methodology or by variation between different parental strains. For instance, we did not find changes in growth of the Δ*kdpD* (Δ*lmo2679*) strain in 6% NaCl, even though its cognate RR KdpE (encoded by *lmo2678*) of *L. monocytogenes* LO28 has been shown to have role under osmotic stress (Kallipolitis and Ingmer, 2001). However, impaired growth of the Δ*kdpE* strain has only been detected when disrupting the *kdpE* gene of *L. monocytogenes* EGD and LO28 strains by insertional mutation (Brøndsted et al., 2003; Kallipolitis and Ingmer, 2001), whilst deletion did not lead to any growth changes in *L. monocytogenes* EGD or EGD-e under osmotic stress (Brøndsted et al., 2003; Williams et al., 2005). This type of

Table 3
Area under curve (AUC) values of *Listeria monocytogenes* wild-type EGD-e and histidine kinase deletion mutant strains.

AUC ± SD[a]						
Strain	42.5 °C	pH 5.6	pH 9.4	6% NaCl	3.5% ethanol	5 mM H_2O_2
EGD-e	7.52 ± 0.65	10.38 ± 0.14	13.10 ± 0.28	8.55 ± 0.75	23.08 ± 0.63	12.28 ± 0.29
Δ*lmo0050* (Δ*agrC*)	6.38 ± 1.15	9.98 ± 0.04*	12.94 ± 0.44	7.11 ± 0.77	21.86 ± 0.27	10.62 ± 0.55*
Δ*lmo0288* (Δ*yycG*)	8.33 ± 0.90	9.57 ± 0.26**	14.43 ± 0.25	9.10 ± 0.38	20.61 ± 0.58*	12.34 ± 0.30
Δ*lmo0692* (Δ*cheA*)	7.21 ± 0.91	9.49 ± 0.14**	12.84 ± 0.56	8.60 ± 0.27	21.51 ± 0.14	12.34 ± 0.29
Δ*lmo1021* (Δ*liaS*)	5.25 ± 0.51*	8.22 ± 0.24**	11.10 ± 0.45**	5.92 ± 0.21**	20.49 ± 0.63*	10.97 ± 0.63*
Δ*lmo1061*	7.70 ± 0.89	9.55 ± 0.24**	13.20 ± 0.23	8.43 ± 0.50	21.57 ± 0.17	12.16 ± 0.33
Δ*lmo1173*	7.56 ± 0.75	9.57 ± 0.25**	13.32 ± 0.16	9.46 ± 0.43	21.90 ± 0.79	12.10 ± 0.22
Δ*lmo1378* (Δ*lisK*)	6.62 ± 1.45	10.03 ± 0.26	13.87 ± 0.33	9.91 ± 0.64	24.56 ± 0.10	11.80 ± 0.19
Δ*lmo1508*	8.11 ± 2.37	9.57 ± 0.23**	14.77 ± 0.17	10.31 ± 0.93	22.67 ± 0.04	12.17 ± 0.29
Δ*lmo1741* (Δ*virS*)	3.19 ± 0.09*	9.63 ± 0.26*	13.57 ± 0.16	9.86 ± 0.99	18.59 ± 0.81*	12.47 ± 0.35
Δ*lmo1947* (Δ*resE*)	7.10 ± 0.40	9.91 ± 0.23*	13.11 ± 0.29	9.84 ± 0.38	21.04 ± 0.40	11.24 ± 0.25**
Δ*lmo2011*	7.73 ± 0.20	9.75 ± 0.19**	12.93 ± 0.08	9.99 ± 0.72	21.81 ± 0.46	11.88 ± 0.16
Δ*lmo2421* (Δ*cesK*)	6.75 ± 0.42	10.03 ± 0.24	12.93 ± 0.11	10.16 ± 0.23	25.11 ± 0.24	11.83 ± 0.24
Δ*lmo2500* (Δ*phoR*)	6.92 ± 0.58	9.93 ± 0.28	14.24 ± 0.43	9.89 ± 0.74	21.61 ± 0.62	11.17 ± 0.81
Δ*lmo2582*	7.81 ± 0.66	10.36 ± 0.23	12.85 ± 0.42	9.33 ± 0.54	21.70 ± 0.51	11.62 ± 0.18*
Δ*lmo2679* (Δ*kdpD*)	7.12 ± 1.34	10.90 ± 0.09	13.36 ± 0.29	8.81 ± 0.46	22.47 ± 0.52	12.01 ± 0.17

[a] Significantly decreased value (**, P < 0.001; *, P < 0.01) (2-tailed *t*-test) compared to the corresponding value of the wild-type.

phenomenon could also be explained by polar effects of the insertional mutation, as was suggested by Brøndsted et al. (2003). Besides KdpDE, CesK (Lmo2421) did not seem to contribute to heat tolerance in our study, although such a role has been indicated for its cognate RR CesR (Lmo2422) of *L. monocytogenes* LO28 (Kallipolitis and Ingmer, 2001). Similarly, HKs ResE (Lmo1947) and PhoR (Lmo2500) showed no role under ethanol stress, while cognate RRs ResD (Lmo1948) and PhoP (Lmo2501) have been reported to be involved in growth of *L. monocytogenes* EGD-e at high ethanol concentration (Williams et al., 2005). However, considering growth of mutant strains in ethanol and hydroxen peroxide in the present study, the changes were altogether considerably minor (Fig. 2E and F). Thus, 3.5% ethanol and 5 mM H_2O_2 might not intrinsically be stressful enough to bring about clear growth differences between TCS mutants and the wild-type strain. Furthermore, it has been reported that there are variations in pathogenicity of *L. monocytogenes* strains EGD, EGD-e, and 10403S (Bécavin et al., 2014). As there are genomic differences underlying these divergent phenotypes (Bécavin et al., 2014), it implicates that also other phenotypic results gained by using different strains should be interpreted with caution. Hence, conclusions on a behavior of a certain strain can be reliably made only based on experiments on the same strain.

In addition to cognate HKs and RRs showing differing results, we did not detect notable growth changes in any of the stress conditions studied when deleting the *lisK* gene of *L. monocytogenes* EGD-e (Tables 3 and 4), although its role in growth and survival of *L. monocytogenes* LO28 under acid and osmotic stresses has been asserted (Cotter et al., 1999; Sleator and Hill, 2005). In addition to different parental strain, this discrepancy may be explained by differences in study conditions and methods, as deletion in the *lisK* gene lead to impaired growth in 8% NaCl (Sleator and Hill, 2005) and lower survival rate during exponential growth at pH 3.5 (Cotter et al., 1999), as compared to growth measurements in 6% NaCl and at pH 5.6 in our study.

We applied a novel complementation approach by modifying the previously published deletion method, and used the cloning shuttle vector plasmid pMAD for allelic exchange (Arnaud et al., 2004). Our earlier complementation strategy by site-specific integration of the wild-type gene, while being functional in complementation of RNA helicase mutants (Markkula et al., 2012a and b), did not complement TCS mutants of *L. monocytogenes* properly. For example, it failed to restore the phenotype of the Δ*lisK* strain to the wild-type level at low temperature (Pöntinen et al., 2015). The new allelic exchange approach allows the construction of a complemented strain that does not harbor any integrated plasmids.

Table 4
Mean maximum growth rates of *Listeria monocytogenes* wild-type EGD-e and histidine kinase deletion mutant strains.

Maximum growth rate ± SD (OD_{600} units/h)[a]						
Strain	42.5 °C	pH 5.6	pH 9.4	6% NaCl	3.5% ethanol	5 mM H_2O_2
EGD-e	0.059 ± 0.016	0.068 ± 0.001	0.13 ± 0.004	0.006 ± 0.0000	0.06 ± 0.006	0.22 ± 0.007
Δ*lmo0050* (Δ*agrC*)	0.068 ± 0.030	0.072 ± 0.001	0.12 ± 0.003*	0.004 ± 0.0000*	0.05 ± 0.005	0.18 ± 0.004
Δ*lmo0288* (Δ*yycG*)	0.065 ± 0.013	0.062 ± 0.001*	0.20 ± 0.011	0.007 ± 0.0000	0.04 ± 0.003	0.23 ± 0.008
Δ*lmo0692* (Δ*cheA*)	0.056 ± 0.015	0.063 ± 0.001**	0.12 ± 0.003	0.006 ± 0.0000	0.05 ± 0.004	0.21 ± 0.006
Δ*lmo1021* (Δ*liaS*)	0.050 ± 0.015	0.043 ± 0.001**	0.07 ± 0.002**	0.002 ± 0.0000**	0.04 ± 0.003*	0.17 ± 0.004**
Δ*lmo1061*	0.060 ± 0.014	0.062 ± 0.001**	0.13 ± 0.004	0.006 ± 0.0000	0.05 ± 0.004	0.22 ± 0.007
Δ*lmo1173*	0.058 ± 0.014	0.062 ± 0.001*	0.13 ± 0.004	0.008 ± 0.0001	0.05 ± 0.005	0.22 ± 0.007
Δ*lmo1378* (Δ*lisK*)	0.058 ± 0.023	0.065 ± 0.001	0.16 ± 0.005	0.009 ± 0.0001	0.07 ± 0.008	0.22 ± 0.007
Δ*lmo1508*	0.062 ± 0.014	0.063 ± 0.001*	0.24 ± 0.009	0.008 ± 0.0001	0.06 ± 0.005	0.22 ± 0.008
Δ*lmo1741* (Δ*virS*)	0.000 ± 0.003*	0.066 ± 0.002*	0.16 ± 0.006	0.007 ± 0.0000	0.04 ± 0.003*	0.24 ± 0.008
Δ*lmo1947* (Δ*resE*)	0.051 ± 0.014	0.061 ± 0.001*	0.13 ± 0.004	0.008 ± 0.0001	0.05 ± 0.004	0.21 ± 0.007
Δ*lmo2011*	0.051 ± 0.010	0.065 ± 0.001*	0.13 ± 0.004	0.009 ± 0.0001	0.05 ± 0.005	0.22 ± 0.008
Δ*lmo2421* (Δ*cesK*)	0.054 ± 0.015	0.065 ± 0.001	0.13 ± 0.005	0.009 ± 0.0001	0.06 ± 0.009	0.21 ± 0.006
Δ*lmo2500* (Δ*phoR*)	0.050 ± 0.013	0.064 ± 0.001	0.21 ± 0.007	0.009 ± 0.0002	0.05 ± 0.005	0.18 ± 0.004
Δ*lmo2582*	0.073 ± 0.026	0.069 ± 0.001	0.12 ± 0.003*	0.007 ± 0.0001	0.05 ± 0.005	0.21 ± 0.006
Δ*lmo2679* (Δ*kdpD*)	0.064 ± 0.025	0.068 ± 0.001	0.13 ± 0.004	0.006 ± 0.0000	0.05 ± 0.004	0.22 ± 0.007

[a] Significantly decreased value (**, P < 0.001; *, P < 0.01) (2-tailed *t*-test) compared to the corresponding value of the wild-type.

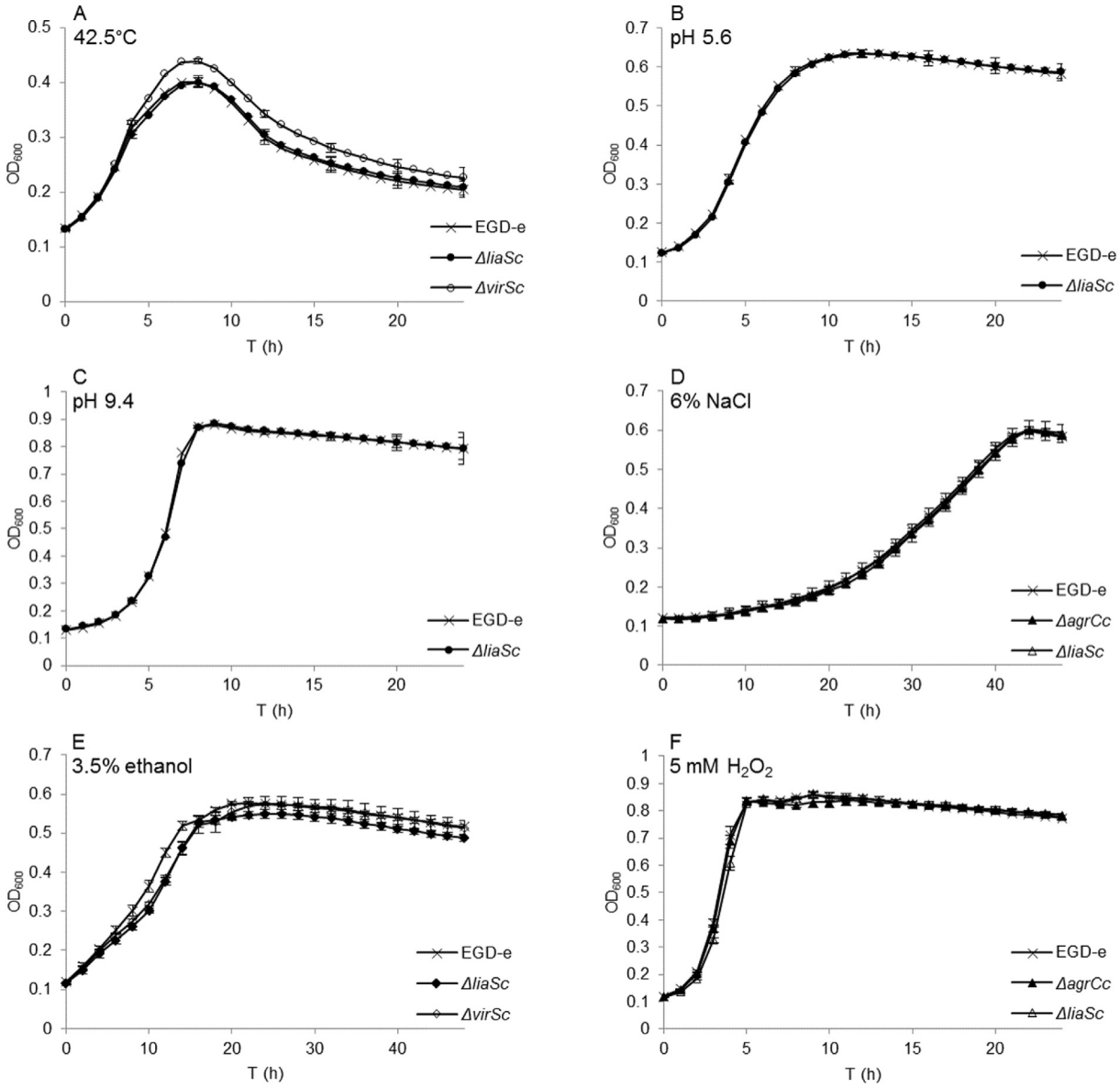

Fig. 4. Growth curves of *Listeria monocytogenes* wild-type EGD-e and the complemented strains (A) Δ*liaSc* and Δ*virSc* in BHI broth at 42.5 °C; (B) Δ*liaSc* at pH 5.6; (C) Δ*liaSc* at pH 9.4; (D) Δ*agrCc* and Δ*liaSc* in 6% NaCl; (E) Δ*liaSc*, and Δ*virSc* in 3.5% ethanol; (F) Δ*agrCc* and Δ*liaSc* in 5 mM H_2O_2. All the strains were grown for 48 h in 6% NaCl and 3.5% ethanol and for 24 h in other stress conditions and the optical density at 600 nm (OD_{600}) was measured at 15-min intervals. The data shown represent the mean OD_{600} values and the error bars the standard deviation of four replicate cultures in 3.5% ethanol and five replicate cultures in other stress conditions.

Consequently, it also eliminates the need for a vector control. Furthermore, the complementation takes place in the target gene locus. This eradicates the possibility that the earlier used complementation using a remote integration site could hamper the perhaps tightly regulated expression of the target gene. In this complementation approach the mutated gene is restored back to the wild-type gene. There is no need for promoter replacements or changes in the flanking regions of the putative operon. Thus, complementation is prone not to cause overexpression of other genes that could alter the resultant phenotype (Cotter et al., 2002). Indeed, this novel approach successfully restored the phenotypes of stress-sensitive mutants to the wild-type level (Figs. 3 and 4A–F).

In conclusion, we identified HKs needed for the growth of *L. monocytogenes* EGD-e under multiple stress conditions that are used for controlling contamination caused by *L. monocytogenes* in food products and food processing environments. We conclude LiaS to play a universal role in stress tolerance of *L. monocytogenes* EGD-e, with most notable impact at 42.5 °C and in 6% NaCl. The results also indicate that complete TCSs AgrAC and VirRS are needed at osmotic and ethanol stresses, respectively. Furthermore, VirS proved significant at 42.5 °C. In addition, we introduced a novel complementation approach for *L. monocytogenes*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2017.01.018.

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